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REPORT

RADIATION-INDUCED ULTRASTRUCTURAL CHANGES IN LYSOSOMES

I. Cytochemical Analysis

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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Atomic Support Agency
Bethesda, Maryland

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RADIATION-INDUCED ULTRASTRUCTURAL CHANGES IN LYSOSOMES

I. CYTOCHEMICAL ANALYSIS

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FOREWORD
(Nontechnical summary)

All cells, with the exception of bacteria and mature red cells are composed of a nucleus and cytoplasm. These two main cell parts contain specialized organelles and structures which function to perpetuate the life of the cell. One of these organelles, the lysosome, is a small cytoplasmic particle containing a variety of enzymes which are capable of breaking down fats, carbohydrates and proteins. Thus the particle has a digestive function in the cell. These lysosomal enzymes, which are bound by the single membrane of the lysosome, can cause damage to the cell if this membrane becomes so altered as to allow their uncontrollable escape into the cytoplasm. The membrane can be made more permeable by treatment with a variety of substances and conditions, which includes radiation. Indeed, enzymes released from radiation-damaged lysosomes could in turn contribute to cellular injury or death.
In the present study a correlation was obtained of the changes which occur in lysosomes after irradiation with the damage which takes place in the cells. The use of an enzyme marker suggested that radiation causes an initial "build-up" of the enzyme within the lysosome 2 hours after irradiation. The possibility exists that this was followed by an enzyme release which could be responsible for cell damage.

ABSTRACT

Ultrastructural and biochemical changes in lysosomes of rat liver following exposure to ionizing radiation were studied. A marker for acid phosphatase was used to visually correlate the progressive changes in lysosomes with the cellular necrobiotic process postirradiation. The earliest observable change in the lysosomes and/or lysosomal enzymes corresponding with the sequence of fine structural alterations following irradiation suggests that radiation labilizes the lysosomal membrane resulting in a release of enzymes responsible for cell damage. The concentration of the lead phosphate reaction product indicated that the initial action on the lysosome is evidently a "build-up" of hydrolytic enzymes within 2 hours after irradiation followed by a gradual release of the marked enzyme 2-24 hours postirradiation as noted by decreased enzyme concentration within the lysosomes. The release of the enzyme appeared to be directly related to an increasing cellular necrobiosis following irradiation.

I. INTRODUCTION

Lysosomes have been identified as distinct intracellular structures bound by a single membrane and containing enzymes. Upon release from the lysosome, these enzymes are capable of causing repairable or irreparable cellular damage.[11] The increase in activity of a number of specific lysosomal enzymes in isolated cells or tissues of irradiated animals has been widely reported.[4,6,7,15,16,22,24] Isolated lysosomes irradiated in vitro showed no distinguishable effects, even at very high doses (1-50 krads).[6] Thus, it has been suggested that the release of the lysosomal enzymes postirradiation was mediated by conditions that require the integrity of the cells.[22] It seemed logical then that clarification on whether or not lysosomes caused damage to irradiated cells would have to be derived from in vivo studies.

An in vivo system to study radiation-induced lysosomal changes has been used by a number of investigators.[2,10,19] More specific studies have been made through the use of a marker for acid phosphatase which made it possible to visually associate the lysosomes with cellular radiation lesions.[3,9,14,18] This test seems to be sufficiently sensitive to detect the differences which prevail between the irradiated and control lysosomal concentration of acid phosphatase.

The objective of the present study is to correlate the progressive changes which occur in lysosomes after irradiation with the postirradiation necrobiotic process which takes place in cells. More specifically, a comparison of the earliest observable change in the lysosomes and/or lysosomal enzyme activity with the sequence of fine structural alteration following irradiation in cells was undertaken.

II. MATERIALS AND METHODS

Eighty Sprague-Dawley rats of the Charles River strain, weighing approximately 200 grams were used. Thirty-two animals were irradiated and the remaining forty-eight animals were used in equal numbers as starved and fed unirradiated controls. The experimental population of rats was exposed to 2 krads of whole-body x radiation from a Maxitron with the following physical parameters: 250 kVp, 30 mA, filtered by 1.2 mm Be and 0.95 mm Cu; HVL - 1.9 mm Cu. The distance of the x-ray tube from the animal midline was 60 cm. For the radiation exposure, all rats of an exposure group were placed in Lucite boxes and arranged in the radiation field so that the tissue dose rate to the midline of the exposure volume was similar for all rats (maximum deviation ± 4 percent).

The irradiated and control animals were anesthetized with 0.25 ml of Sodium Nembutal (50 mg/ml) and the liver perfused with 3.0 percent glutaraldehyde buffered with .067 M cacodylate pH 7.4. The liver specimen was rapidly removed, cut into strips (2 x 5 x 10 mm) and washed overnight at 4°C in 0.1 M cacodylate containing 7.5 percent sucrose. The sections were subsequently mounted in agar and cut into 50 μm sections with a Sorvall tissue sectioner. The sections were incubated for 30 minutes at 37°C in a substrate medium (Gomori method, Barka and Anderson[1]) containing β-glycerophosphate (Grade III, Sigma Chemical Co., St. Louis, Missouri) pH 5.0. The liver specimens, used as controls for those incubated in the substrate medium, were incubated in the medium minus β-glycerophosphate.

The sections were washed in Tris buffer containing 7.5 percent sucrose, pH 5.0 following the incubation procedure. The sections were then postfixed in 1 percent

osmium tetroxide,[12] dehydrated in graded ethanol solutions and embedded in Maraglas.[23] The blocks were cut with a Porter-Blum MT2 ultramicrotome and sections mounted on uncoated grids. After staining with uranyl acetate[20] and lead citrate[17] the sections were examined in a Siemens electron microscope.

III. RESULTS

An electron micrograph of a normal hepatic lysosome is illustrated in Figure 1. In comparison, lysosomes incubated in acid phosphatase substrate medium show lysosomal aggregates in the vicinity of the bile canaliculus with electron dense areas of the reaction product, lead phosphate (Figure 2). The lysosomes (L) are intact with single membranes, showing some variation in the internal structure and number. The areas of lead salt deposits, usually spherical in nature, are normally uniformly dense and eccentrically located in the lysosomes. Exceptions to this are illustrated

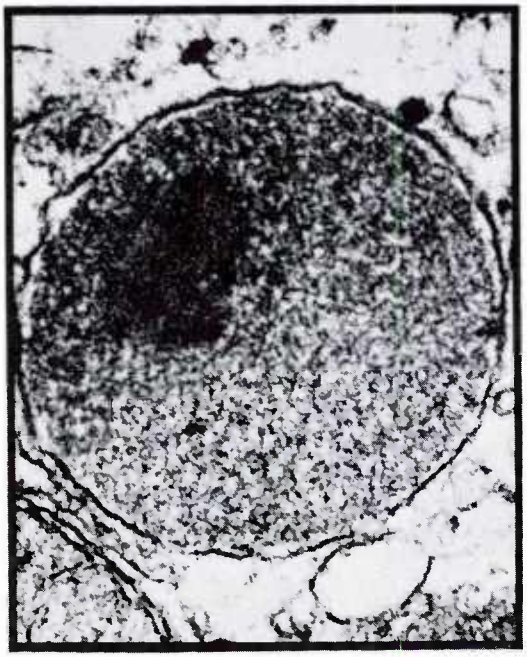

Figure 1. Lysosome of a normal rat liver cell. X 100,000

in Figure 2b, in which a dark area can be seen with a very light matrix and in Figure 2c (arrow) in which the reaction product can be seen as a fibrous material.

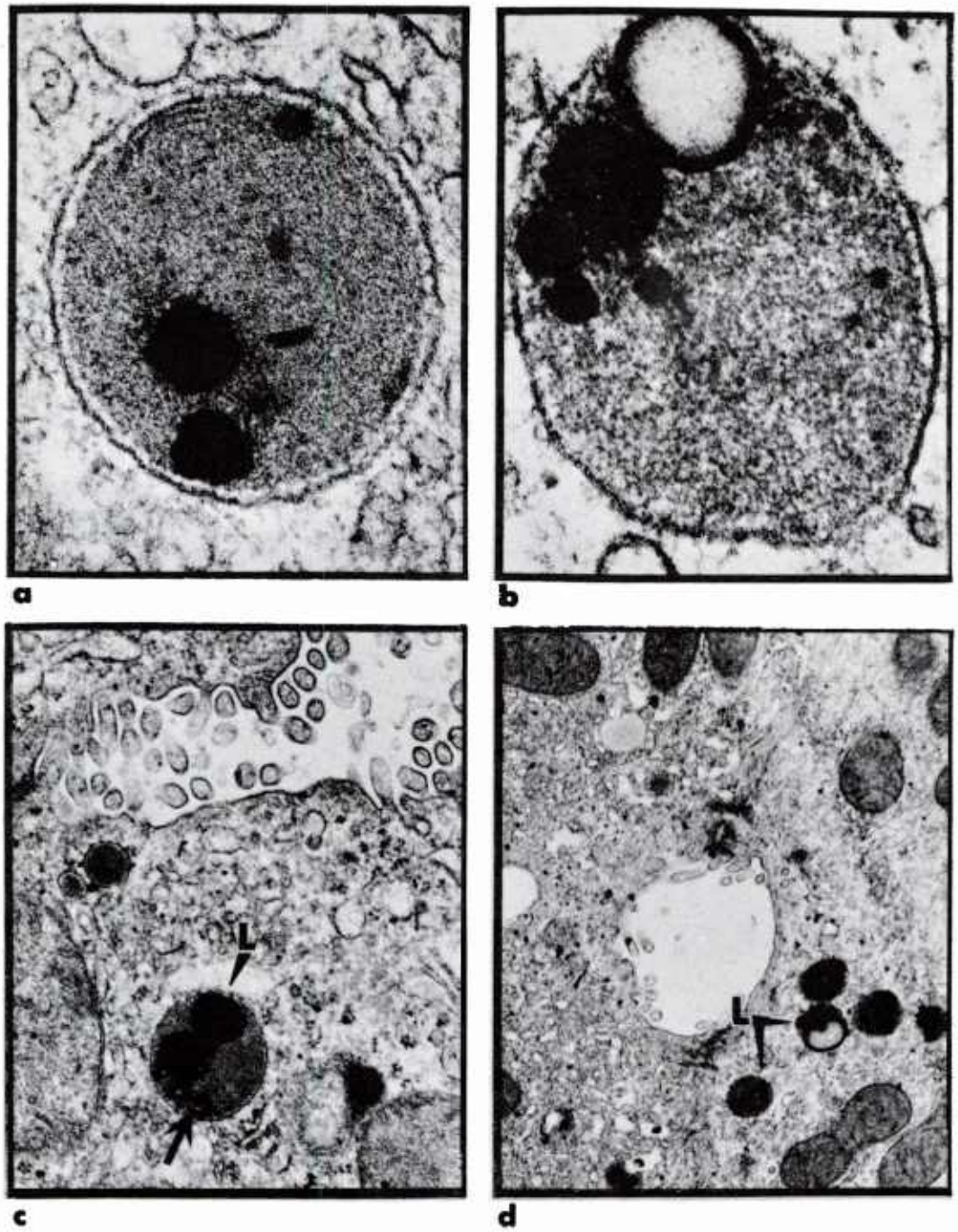

Figure 2. Normal lysosomes of rat liver cells incubated in β-glycerophosphate. The electron dense areas indicate the presence of acid phosphatase. (a, b, c, and d present different sections of the same specimen.) (a) X 110,000; (b) X 115,000; (c) X 32,500; (d) X 16,250

There was no difference between the starved and fed unirradiated controls and they were used interchangeably. The hepatocytes of animals sacrificed immediately after irradiation (1-2 hours) exhibited little change in morphology when compared to the normal cells (Figure 3). The reaction product of the lysosomes in some instances appeared to be more dense and covered a larger area. Some of these areas almost completely covered the whole lysosome (Figure 3a). In other cells, the reaction product was more fibrous or appeared in greater numbers of individual inclusion (Figure 3c and d). The fine structure of the cell appeared to be normal in all other aspects. The hepatocytes of the animals sacrificed 24 hours after irradiation were morphologically similar to the hepatocytes of the animals sacrificed earlier. The lysosomes of these two groups of animals were very much alike, although there appeared to be a decrease in the concentration of the reaction product (Figure 4). The fine structure of the hepatocytes gave initial evidence of cellular necrosis and the appearance of glycogen (G).

The electron micrographs of the specimen collected 48 hours after irradiation began to show marked changes in fine structure (Figure 5). The most apparent changes were: (1) the appearance of multivesicular bodies; (2) the formation of myelin-like figures in and around prominent glycogen areas (rosettes); (3) the reduction and quite often the absence of electron dense areas of reaction product in most lysosomes. This was accompanied with evidence of membrane degradation and a clearing of the lysosomes.

The most striking change in the fine structure of the hepatocytes of the animals 72 hours after irradiation is illustrated in Figure 6. The lysosomes exhibited

marked evidence of deterioration with membrane budding, breaking and matrix clearing. A few lysosomes still had dense peripheral areas. In some areas the lysosomes could be seen as aggregates, especially in the necrobiotic areas. The material which composed its matrix gave the morphological appearance of glycogen (Figure 6b). Some of the lysosomes which gave no evidence of the presence of the reaction product assumed the role of autophagic vacuoles (Figure 6a, b, c). The cytoplasm showed evidence of a reduced amount of glycogen and extreme deterioration of fine structure. Areas of the rough endoplasmic reticulum were without granules in some instances. The mitochondria did not show evidence of damage. The mitochondrial granules and membranes appeared normal except those externally associated with myelin-like figures (arrow) where degradation of the mitochondria occurred (Figure 6d).

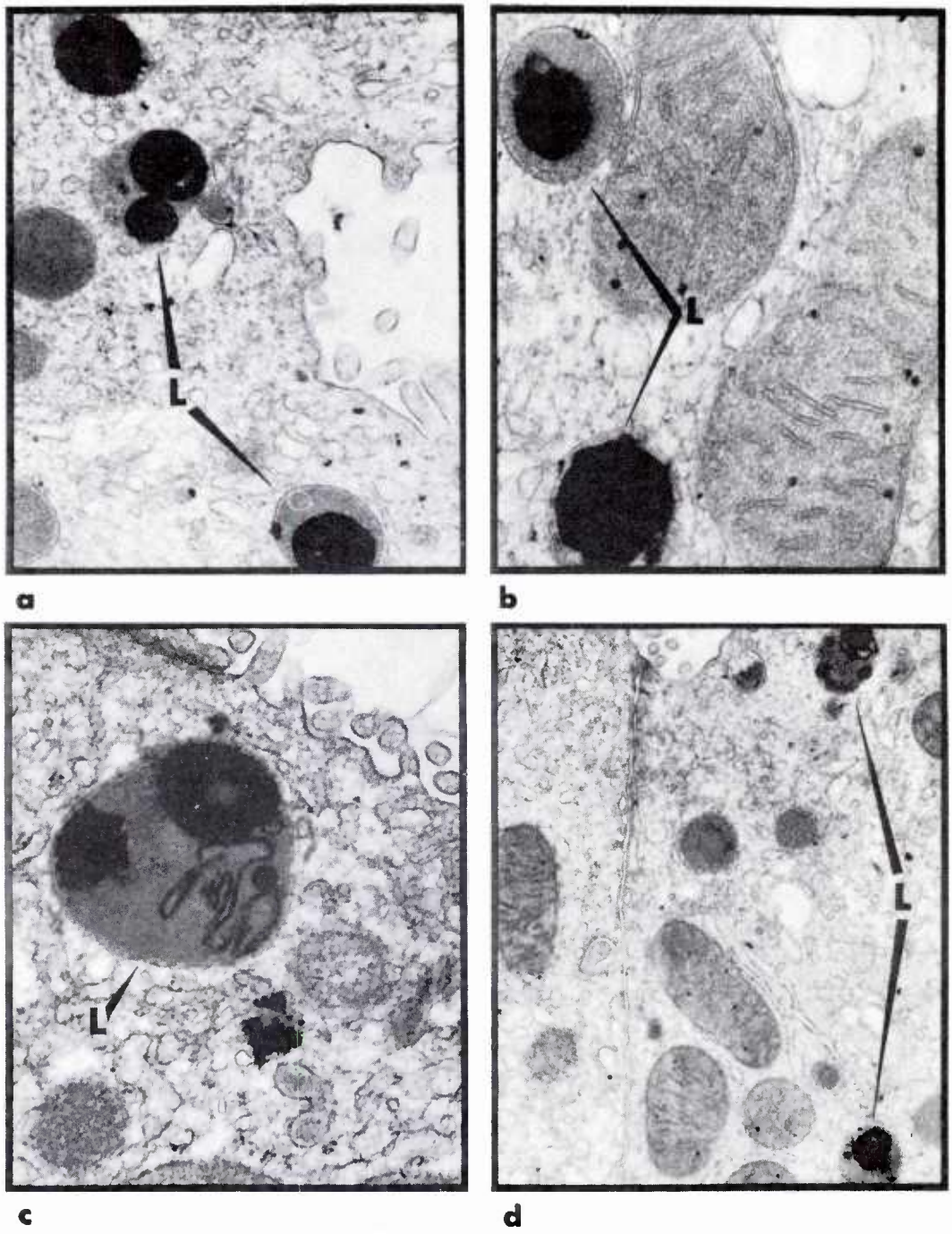

Figure 3. Lysosomes of liver cells of rats sacrificed 2 hours after exposure to 2 krads of x rays. The liver specimen was incubated in β-glycerophosphate to indicate the presence of acid phosphatase. (a, b, c, and d present different sections of the same specimen.) (a) X 31,200; (b) X 49,000; (c) X 40,000; (d) X 20,000

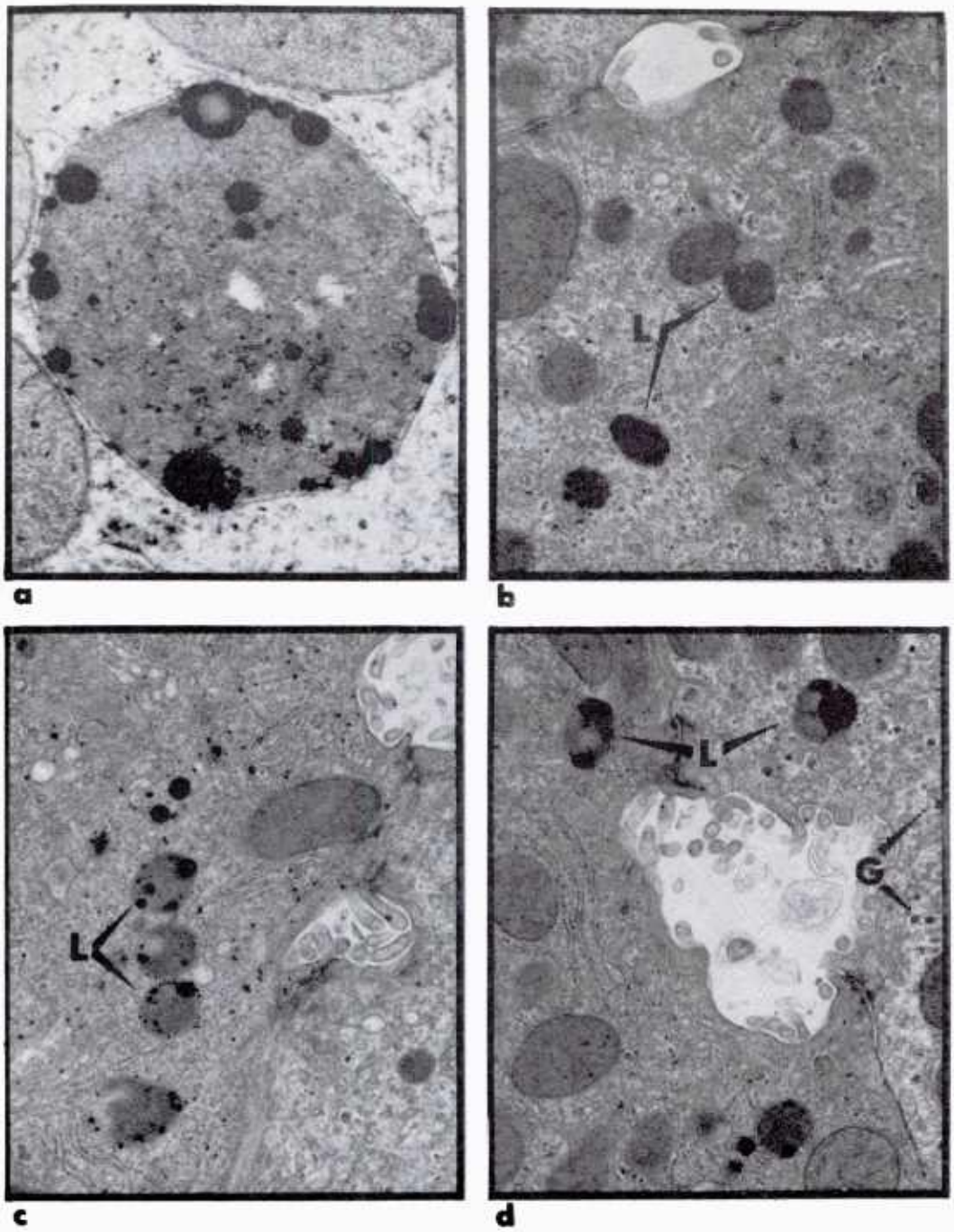

Figure 4. Lysosomes of liver cells of rats sacrificed 24 hours after exposure to 2 krads of x rays. The liver specimen was incubated in β-glycerophosphate to indicate the presence of acid phosphatase. (a, b, c, and d present different sections of the same specimen.) G = glycogen. (a) X 42,500; (b) X 21,250; (c) X 21,250; (d) X 21,250

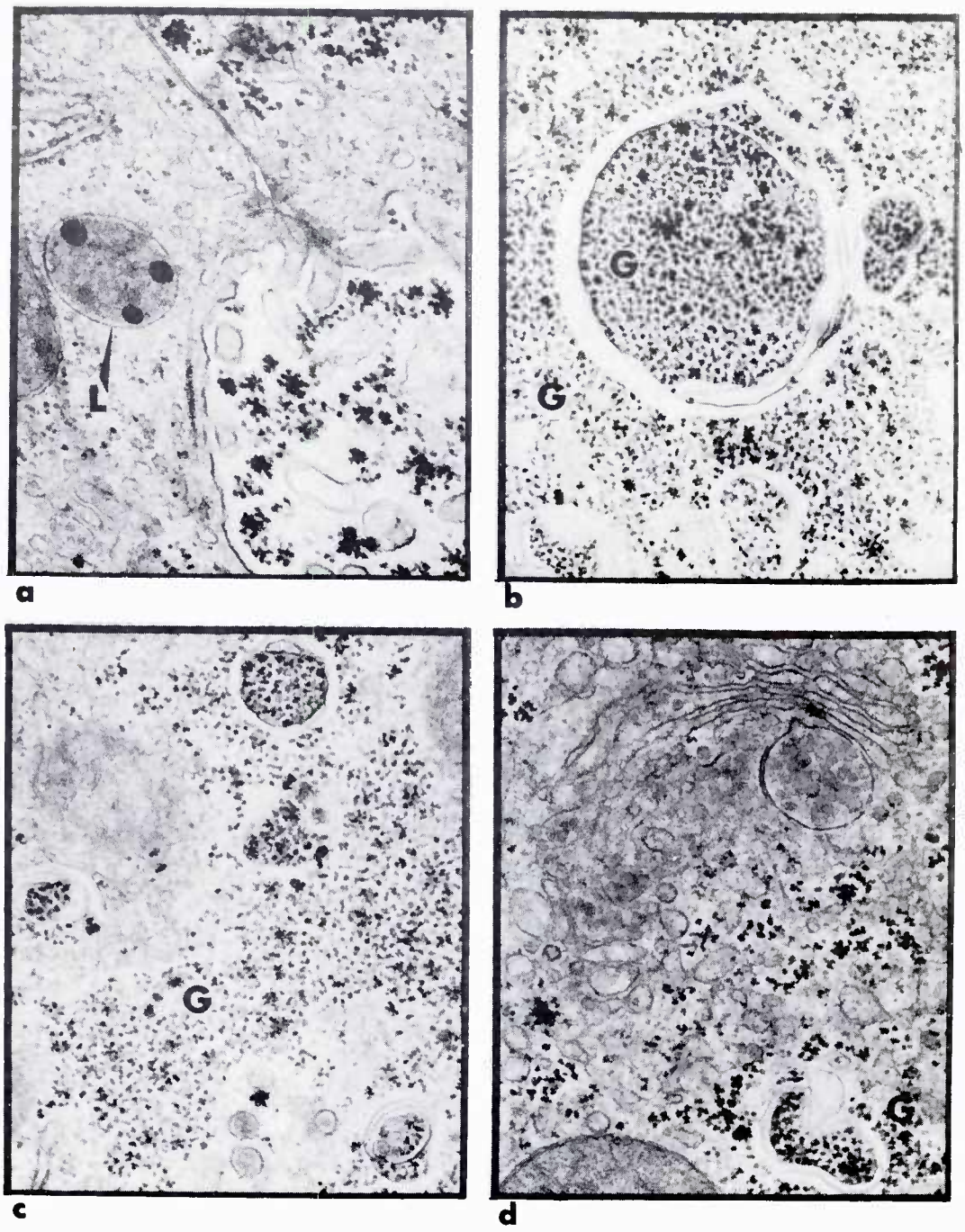

Figure 5. Specimen of liver cells of rats sacrificed 48 hours after exposure to 2 krads of x rays. The liver specimen was incubated in β-glycerophosphate to indicate the presence of acid phosphatase. (a, b, c, and d present different sections of the same specimen.) G = glycogen. (a) X 42,500; (b) X 37,500; (c) X 37,500; (d) X 37,500

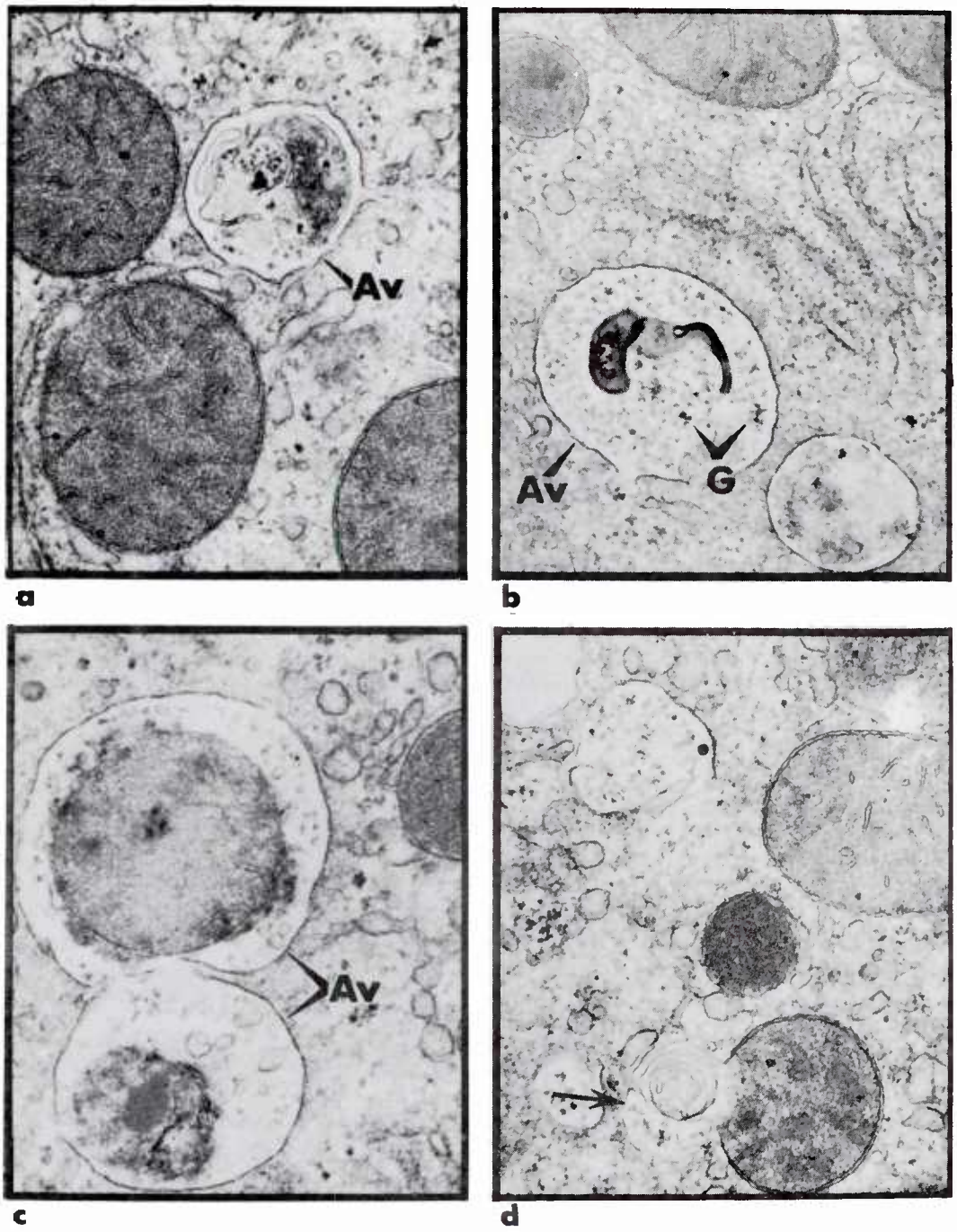

Figure 6. Specimen of liver cells of rats sacrificed 72 hours after exposure to 2 krads of x rays. The liver specimen was incubated in β-glycerophosphate to indicate the presence of acid phosphatase. (a, b, c, and d present different sections of the same specimen.) Av = autophagic vacuoles. (a) X 37,500; (b) X 37,500; (c) X 37,500; (d) X 37,500

IV. DISCUSSION

It has been suggested that if lysosomes were the cause of radiation injury in cells then we should find some changes in the lysosomes occurring before the onset of a visible cellular injury.[21] The results of the present cytochemical study indicated that lysosomes of hepatocytes, under the experimental conditions described, are susceptible to radiation effects which are observable within 2 hours after irradiation (Figure 7). This, however, was manifested as an increase in hydrolytic enzyme activity (reaction product, lead phosphate) and not observed as an alteration in lysosomal fine structure nor was it accompanied by any visible changes in the cytoplasmic ultrastructure. This early increase in enzyme activity has been reported by others. In some cases this was observed as early as 30 minutes postirradiation[9] and in other instances not until 16 hours postirradiation.[19] The significance of this "build-up" phenomenon is not yet known. It has been reported, however, that frog neuronal lysosomes showed variable deterioration (swelling and clearing) after exposure to 1000-2000 R x rays, which is probably due to an increased permeability of the lysosomal membrane, causing entry of fluids.[13] Pronounced escape of lysosomal enzymes into the cytoplasm was questionable.

Evidence for enzyme release was reported by Brandes et al.,[3] who observed the presence of the reaction product in the cytoplasm and in intercellular spaces 3 days after exposure to radiation. However, enzymes could be released and might not be detected in the cytoplasm. As a matter of fact, the presence of a concentrated form of reaction product outside the lysosomes within the matrix of the cytoplasm at any time after irradiation was not expected in the present study. The enzyme

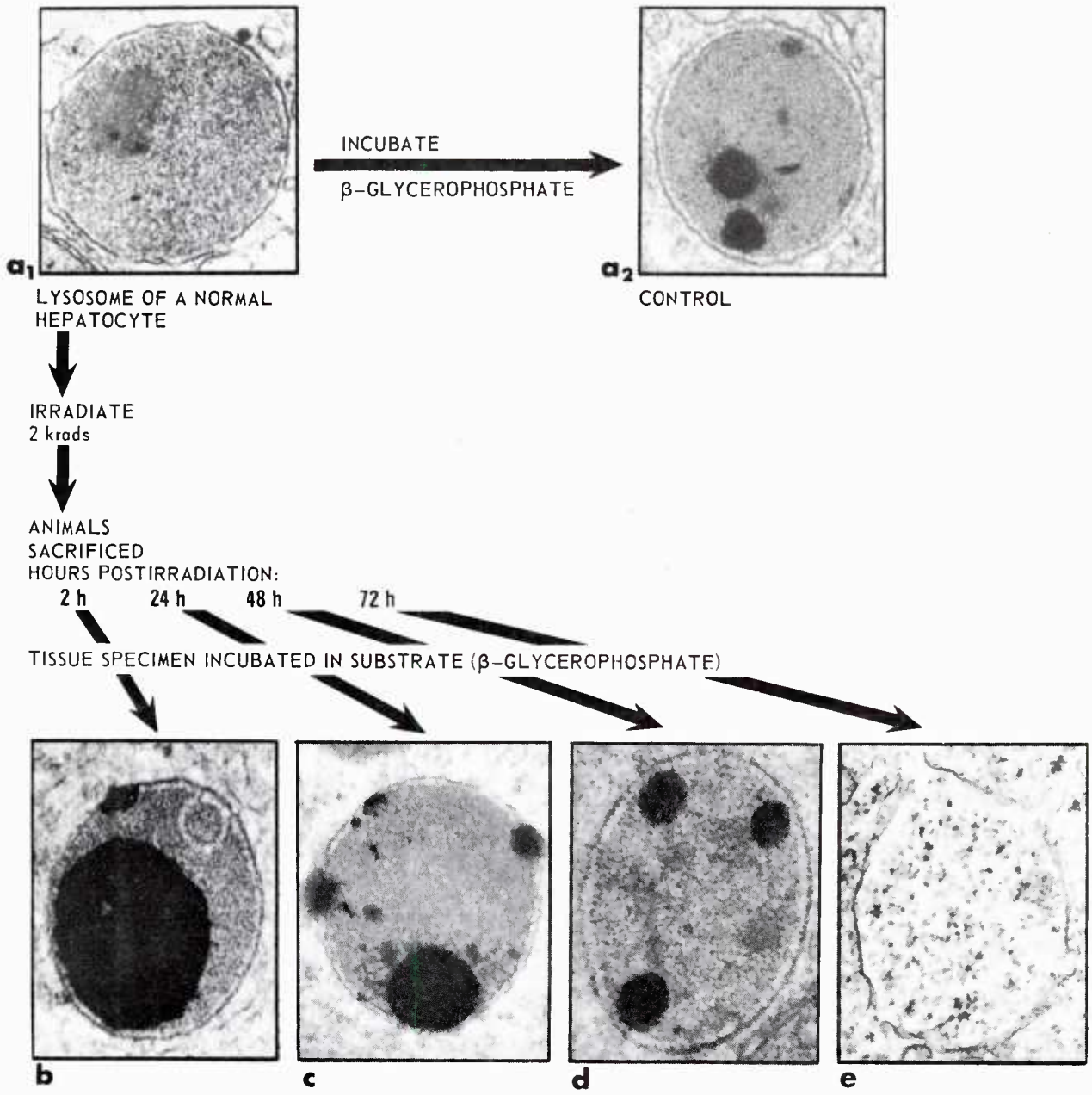

Figure 7. Schematic representation of lysosomal changes following x irradiation. a_1 is a lysosome of a normal hepatocyte. a_2 is a lysosome of a normal hepatocyte after incubation in the substrate medium β-glycerophosphate. b, c, d, and e represent lysosomes at various periods after irradiation, 2, 24, 48 and 72 hours, respectively. At b there is a "build-up" of reaction product which decreases at c and d. There is a loss of membrane competence 72 hours after irradiation (e) allowing the free movement of material across the membrane, resulting in a lysosomal clearing phenomenon.

upon leaving the relatively small lysosomal particle and entering the cytoplasmic matrix is immensely diluted and would not necessarily be visible as dense lead salt deposits.

Alterations in the fine structure of the cell within 2 hours after irradiation were not apparent. Ultrastructural alterations have been identified within parenchymal cells of liver tissue as early as 2 minutes after exposure[8] where the doses used were up to 16 krads. That study could not be correlated with the present study because of the significant difference in the doses used.

The number and size of lysosomes do not show a notable change 24 hours after irradiation, yet, there was a decrease of the reaction product (Figure 7). This condition could be the result of a release of lysosomal enzymes in view of the fact that we also began to observe signs of cellular atrophy which were especially prominent in specimens collected 48 hours after exposure. The appearance of glycogen cannot be ascribed to an injury phenomenon. The rosettes are more evident in the liver of animals that are briefly fasted or subjected to other experimental treatments which result in the depletion of carbohydrate stores.[5] The abundance of glycogen, therefore, could be ascribed to inanition in the animals with severe radiation sickness. The appearance of annuli surrounding areas of dense glycogen rosettes can only be explained in terms of their relationship with an overall deterioration of the cell. Annuli apparently formed from the endoplasmic reticulum were reported in liver cells as early as 2 minutes after exposure,[8] but were not observed in our specimen until 48 hours after exposure. The clearing of lysosomes and breakdown of their membranes, apparent by the 3rd day after exposure, was probably the

most convincing evidence of a possible release of lysosomal enzymes capable of breaking down all the major constituents of the cell.

The formation of radicals is a well-known effect of radiation on an aqueous environment such as the cell. It has been suggested that irradiation causes the formation of lipid peroxides in lysosomes which leads to rupture of the lysosomal membrane and allows the release of the hydrolytic enzymes.[24] It has also been suggested that the formation of free radicals is mediated through a combination of lipid and oxygen which act as free radical initiators to form unstable peroxides. Lysosomes are particularly labile to peroxides.[21] The lysosomal membrane was found extremely labile when isolated and subjected to hydrogen peroxide.[4] In addition to the proposed effect of free radicals, Rahman[15] suggested that the action on the membrane was mediated through a hormonal action which occurs in vivo. A subsequent test of this theory with a thyrotropic hormone was positive.[16] Perhaps this is what Sottocasa[22] had in mind when he suggested that the release of the enzymes required the structural integrity of the cell.

The selective entry of fluids could explain the increase in size of lysosomes after irradiation reported by Brandes et al.[3] and Pipan.[14] The increase in the number of lysosomes could not be explained, except perhaps radiation has a stimulatory effect on the sites of lysosomal formation. The many small lysosomes could be newly formed ones.

In the present report, it is suggested that free radicals are formed in the hepatocytes immediately following irradiation which labilizes the lysosomal membrane and makes it more permeable. To what extent hormones are involved is not understood.

Initially there is a build-up of hydrolytic enzymes in the lysosomes. This phenomenon was reported by Pick[13] and is viewed as an activation of hydrolytic enzymes followed by a gradual selective release which becomes apparent after 2 hours postirradiation. This eventually leads to cellular necrobiosis.

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Ultrastructural and biochemical changes in lysosomes of rat liver following exposure to ionizing radiation were studied. A marker for acid phosphatase was used to visually correlate the progressive changes in lysosomes with the cellular necrobiotic process postirradiation. The earliest observable change in the lysosomes and/or lysosomal enzymes corresponding with the sequence of fine structural alterations following irradiation suggests that radiation labilizes the lysosomal membrane resulting in a release of enzymes responsible for cell damage. The concentration of the lead phosphate reaction product indicated that the initial action on the lysosome is evidently a "build-up" of hydrolytic enzymes within 2 hours after irradiation followed by a gradual release of the marked enzyme 2-24 hours postirradiation as noted by decreased enzyme concentration within the lysosomes. The release of the enzyme appeared to be directly related to an increasing cellular necrobiosis following irradiation.

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